JOURNAL OF BACTERIOLOGY, Jan. 2002, p. 596–599 Vol. 184, No. 2
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.2.596–599.2002

epr Is Transcribed from a σ^D Promoter and Is Involved in Swarming of *Bacillus subtilis*

Madhulika Dixit,† Charuta S. Murudkar, and K. Krishnamurthy Rao*

Biotechnology Centre, Indian Institute of Technology, Powai, Mumbai 400076, India

Received 14 August 2001/Accepted 20 October 2001

Epr is a minor extracellular protease secreted by *Bacillus subtilis* 168. In this study, we show that *epr* is transcribed by Eσ^D, the RNA polymerase associated with transcription of genes involved in chemotaxis and motility. Disruption of *epr* abolished swarming of *Bacillus subtilis*, suggesting its involvement in motility.

At the onset of stationary phase, *Bacillus subtilis* secretes at least seven extracellular proteases of which subtilisin (*aprE*) and a neutral metalloprotease E (*nprE*) are the most abundant (6). Of the remaining five minor extracellular proteases, Epr (*epr*) contributes 2 to 4% to this pool (18). The expression of these enzymes is stringently regulated, as best exemplified by the *aprE* gene (23). However, these proteases appear to be dispensable for growth (11, 19), and their relevance in the physiology of the cell remains to be determined.

The transition state is also characterized by the acquisition of specific functions such as competence and motility (23). The expression of genes related to motility, chemotaxis, and flagellar assembly is governed by the alternate sigma factor σ^D (14). σ^D complexed to the core RNA polymerase (Eσ^D) recognizes and binds the bipartite promoter sequences 5′-CTAAA-3′ (−35) and 5′-CCGATAT-3′ (−10) (14). We present here evidence that *epr* is transcribed from a σ^D-dependent promoter and that this gene is involved in the swarming of *B. subtilis*.

Although *epr* has been cloned and sequenced (4, 18), the identity of its promoter and the possible physiological role(s) of this gene have so far not been elucidated. To identify the promoter for *epr*, we examined the DNA sequence upstream of the ribosome-binding site (RBS) for a putative bipartite promoter sequence that may be related to known promoter sequences for the different sigma factors in *B. subtilis*. A σ^D-type promoter with a −35 (CTATT) and a −10 (CCGATAT) element was identified (Fig. 1) that showed matches of 3 of 5 and 7 of 7, respectively, with the consensus σ^D promoter (14) and an optimal spacing of 17 bp between the two elements. To determine that indeed this promoter was utilized, primer extension analysis was employed to identify the *epr* transcription start site. Total RNA was isolated from *B. subtilis* 168/pIC56-4 and 1A716/pIC56-4 carrying the *epr* gene in a multicopy plasmid, pIC56-4 (Table 1). Since the levels of Epr are very low (4, 18), we were unable to detect *epr* mRNA from a single-copy gene and hence we have used a multicopy vector. A polyacrylamide gel electrophoresis-purified primer, KKR50 (5′-CGAG GATCCTGTACAACAAGTTTGCA-3′) (Fig. 1), end labeled with [γ-^{32}P]ATP (5,000 Ci/mM) and T4 polynucleotide kinase, was annealed with 10 μg of RNA, and the primer was extended with Moloney murine leukemia virus reverse transcriptase (1, 17). The extended product(s) was analyzed on a 7 M urea–8% polyacrylamide denaturing gel. To obtain the template for the sequencing reactions, a DNA fragment of 360 bp that overlapped the putative start site was obtained from pIC56-4 by PCR amplification with the primers KKR76 (5′-CGAGATCT CTGCAGTTTTCCCGGCGAC-3′) and KKR44 (5′CTGGAT CCAGGGGGCTGAAAAACAGAGTGAC-3′) (Fig. 1) that carry *Pst*I and *Bam*HI restriction sites, respectively. The amplified product was cloned in M13mp19, and single-stranded circular DNA was isolated (17) and used as a template in the sequencing reactions with end-labeled KKR50 and Sequenase version 2.0 (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Figure 2 (lane 1) shows two extended products for RNA derived from 168/pIC56-4. The extended products correspond to a C (plus strand) and an A (plus strand) located six and seven bases downstream of the −10 position of the putative σ^D promoter. This result demonstrates that *epr* transcription is governed by the σ^D promoter. No extended products were obtained for RNA derived from 1A716/pIC56-4 (Fig. 2, lane 2), in which *sigD* is mutated, indicating that transcription of *epr* occurs only when σ^D is present. The relative intensities of the two extended products were similar, suggesting that the two RNA transcripts are made in equal proportion. Alternatively, one of the products which terminates at A may have arisen as a result of premature termination during reverse transcription.

To further confirm that the *epr* gene is transcribed by Eσ^D, the promoter activity of *epr* was determined in both *B. subtilis* 168 and 1A716 containing the σ^D promoter, fused to the β-galactosidase gene of *E. coli*, in plasmid pRB381 (3). A 250-bp DNA segment (U_7) containing the σ^D promoter, the RBS, and the ATG was PCR amplified from pIC56-4 with the primers KKR77 (5′-CGAGATCTCTGCAGGCTCATCTTAAAAAC C-3′) and KKR36 (5′-CTTAGGATCCATGATTCATCTCC-3′) (Fig. 1) that contain restriction enzyme sites for *Pst*I and *Bam*HI, respectively. The amplified product was digested with *Pst*I-*Bam*HI, cloned in pRB381 to give $pRBU_7$, and transformed in *B. subtilis* 168 and 1A716 to give 168/$pRBU_7$ and 1A716/$pRBU_7$, respectively. The cells were grown at 37°C in Penassay broth to stationary phase (i.e., to an optical density at

* Corresponding author. Mailing address: Gene Regulation Lab, Biotechnology Centre, Indian Institute of Technology, Powai, Mumbai 400076, India. Phone: (91) (22) 576-7776. Fax: (91) (22) 572-3480. E-mail: kkr@helix.btc.iitb.ac.in.

† Present address: Department of Physiology, College of Medicine, University of Tennessee Health Science Center, University of Tennessee, Memphis, TN 38163.

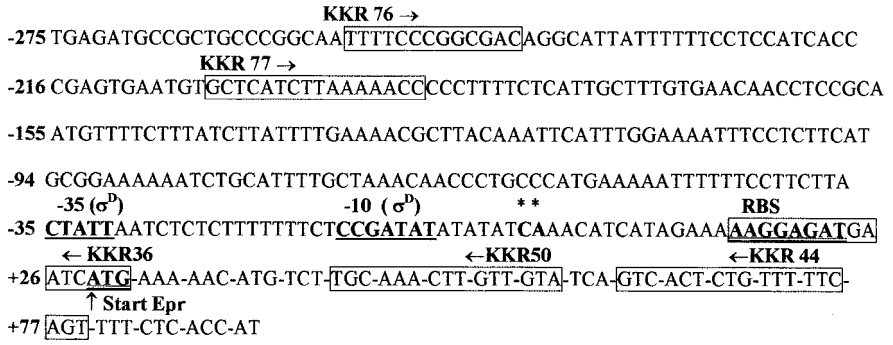

FIG. 1. Sequence of *epr* (4, 18) (accession no. X73124) with positions of the primers used in the study. The transcription start sites are marked with asterix. C* has been denoted as +1.

600 nm [OD_{600}] of ~2.0), and the β-galactosidase activities were determined (15). The enzyme activity in 168/pRBU_7 was observed to be 4,860 Miller units, whereas no activity was seen in 168/pRB381, indicating that the 250-bp region in pRBU_7 carries the *epr* promoter. However, the β-galactosidase activity in 1A716/pRBU_7 that lacks σ^D was completely abolished. Thus, the results of the primer extension analysis and the reporter gene assays clearly demonstrate that transcription of *epr* is σ^D dependent.

Among the many functions governed by σ^D in *B. subtilis*, several of these genes relate to chemotaxis and to motility that includes flagellin biosynthesis (5, 14). We thus decided to investigate whether Epr may be involved in either chemotaxis or motility. Swarming of bacterial cells is one of the several motility related functions involving surface translocation on soft agar that is accompanied by changes in cell shape and hyperflagellation (9). In nature, swarming provides a means for colonization of bacteria to achieve an ecological niche (8). Proteolysis appears to be one of the mechanisms that regulate the differentiation of the swarmer cells. For example, in the swarming of *Vibrio paraheamolyticus*, the LonS protease, which is functionally homologous to *E. coli* Lon, regulates cell elongation and lateral flagellum expression (7, 22). Mutants that lack LonS have a constitutive elongated phenotype. Attempts to demonstrate a "swarm$^+$" phenotype in *lonS* mutants were unsuccessful (22). We were thus interested in determining whether there was a relationship between the swarming of *B. subtilis* and *epr*. Swarm assays were carried out with *B. subtilis* 168, 168Δ*epr*, and 168Δ*epr*/pIC216-*epr* (Table 1). The cells were grown in Luria-Bertani (LB) broth at 37°C to an OD_{600} of ~4.0, and 1-μl portions of the cultures were spotted onto LB agar plates containing 0.8% agar. The plates were incubated at 37°C for 24 h. The results (Fig. 3) clearly show that the wild-type *B. subtilis* 168 (Fig. 3a) exhibited swarming on soft agar plates, whereas 168Δ*epr* (Fig. 3b) did not. The ability of 168Δ*epr* to swarm was restored (Fig. 3c) when a functional *epr* gene was introduced (168Δ*epr*/pIC216-*epr*). It is important to distinguish here between swimming and swarming motility. Swimming motility is exhibited by individual cells, does not involve communication with other cells, and is observed in liquid medium as well as on plates containing low percentages of agar (0.2 to 0.4%) (8). Swarming, on the other hand, is exhibited on plates containing higher percentages of agar (>0.5%) and requires close cell-to-cell contact, and signaling

TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Genotype[a]	Source or reference
Plasmids		
pIC56-4	Plasmid pIC56 (Em^r) (20) bearing *epr* gene at the *Bam*HI site	Laboratory Stock (16)
pIC216-*epr*	Plasmid pIC216 (Sp^r) (21) bearing *epr* gene at the *Sal*I site; the *epr* gene was excised as a *Sal*I fragment from pIC56-4	This study
pRB381	*E. coli-B. subtilis* shuttle vector for translational fusion with β-galactosidase gene	*Bacillus* Genetic Stock Center (3)
pRBU_7	pRB381 bearing a 250-bp (−205 to +31 from transcription start site of *epr*) insert containing the *epr* promoter region, RBS, and ATG in translational frame with the *lacZ* gene	This study
Strains		
168	*trpC2*	*Bacillus* Genetic Stock Center
1A716	*sig*D::pLM5 Cm^r *trpC2*	*Bacillus* Genetic Stock Center
168Δ*epr*	*trpC2 epr*::Em^r	Laboratory stock (16)
168/pIC56-4	*B. subtilis* 168 bearing pIC56-4	This study
1A716/pIC56-4	*B. subtilis* 1A716 bearing pIC56-4	This study
168/pRB381	*B. subtilis* 168 bearing pRB381	This study
168/pRBU_7	*B. subtilis* 168 bearing pRBU_7	This study
1A716/pRBU_7	*B. subtilis* 1A716 bearing pRBU_7	This study
168Δ*epr*/pIC216-*epr*	*B. subtilis* 168Δ*epr* bearing pIC216-*epr*	This study

[a] Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance. Numbers in parentheses are references.

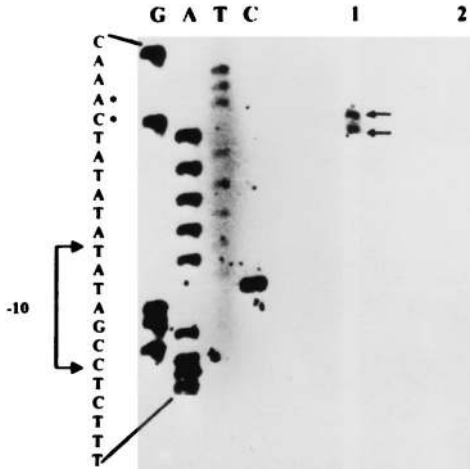

FIG. 2. Primer extension analysis of RNA from 168/pIC56-4 (lane 1) and 1A716/pIC56-4 (lane 2). Autoradiography exposure was for 72 h. Start sites are marked with dots.

of information between individual cells is an integral part of this phenomenon (8). Microscopic observation of 168Δ*epr* reveals that it is motile and that the vigor of its motility is comparable to that of the wild type. Motility assays performed with *B. subtilis* 168 and 168Δ*epr* on LB agar plates containing increasing concentrations of agar (0.3 to 0.8%) revealed that disruption of *epr* did not affect the spreading of the cells at up to 0.5% agar (swimming) but that the cells were incapable of spreading on plates containing 0.8% agar (swarming) (data not shown). We thus believe that this phenomenon is swarming motility and that Epr is involved in the swarming of *B. subtilis.* It is possible that Epr may be involved in providing signals to mediate cell-cell communication, a property of swarmer cells. Communication between cells in processes such as quorum sensing and competence appears to involve peptides that are

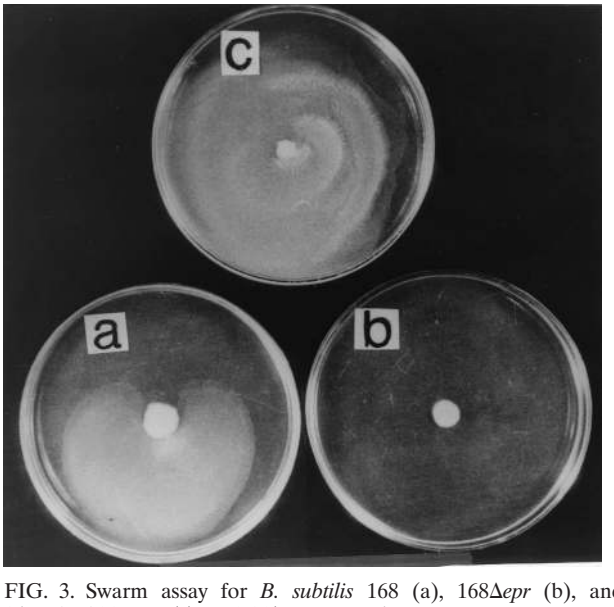

FIG. 3. Swarm assay for *B. subtilis* 168 (a), 168Δ*epr* (b), and 168Δ*epr*/pIC216-*epr* (c) on 0.8% LB agar plates.

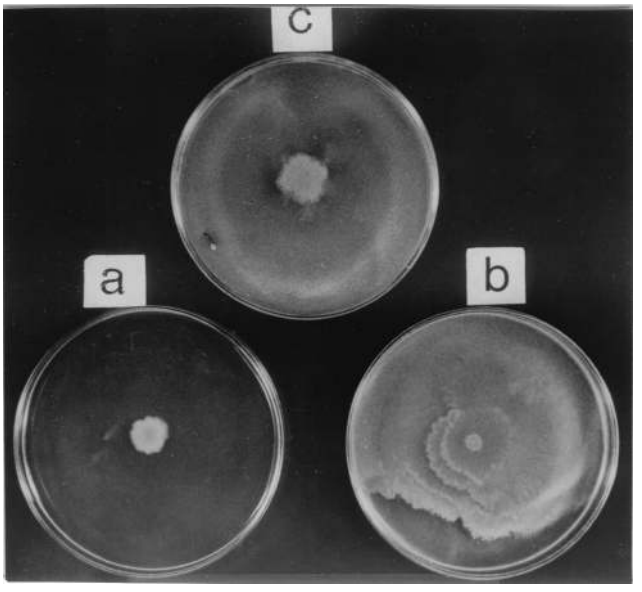

FIG. 4. Swarm assay for *B. subtilis* 168 (c) and 168Δ*epr* (a) on a 0.8% LB agar plates and 168Δ*epr* on a 0.8% conditioned LB agar plate (b).

secreted and reimported (2, 13). We propose that the role of Epr in swarming motility is to provide the signal(s) utilizing its proteolytic activity, or Epr itself may be proteolysed by other proteases to generate the signal(s). In support of the above hypothesis, swarm assays were performed with 168Δ*epr* on 0.8% LB agar plates as well as on 0.8% conditioned LB agar plates (i.e., containing a 1:1 mixture of fresh LB and spent medium supernatant derived from *B. subtilis* 168, grown to an OD_{600} of ~4.0 and filter sterilized). The results in Fig. 4 demonstrate that the 0.8% conditioned LB agar plate supported the swarming of 168Δ*epr* (Fig. 4b), whereas it was unable to swarm on a 0.8% LB agar plate (Fig. 4a). This clearly indicates that a signal released from wild-type cells into the medium supported the swarming of 168Δ*epr* that was otherwise missing in the mutant, thus implicating Epr in the signaling process for swarming. Work is in progress to determine the identity of the signal and the signaling mechanism. Epr is also known to exist in multiple forms due to posttranslational processing. One such product of Epr is a C-terminal domain of 240 amino acids that is lysine rich and whose function is unknown (4, 18). Intriguingly, a homology search of this domain with BLASTP in GenBank (http://www.ncbi.nlm.nih.gov) showed considerable homology with various isoforms of tropomyosin across eukaryotes. Tropomyosins are known to regulate actin-myosin interactions in eukaryotes (12). Recently, actin homologs have been identified in *B. subtilis* that are responsible for maintaining cell shape (10). It may well be that an additional role of Epr is to regulate morphologic changes such as the cell elongation that accompanies swarming.

This work was supported by the Department of Science and Technology (SP/SO/D-58/96) and the Council of Scientific and Industrial Research [37(950) 97-EMR-II] to K.K.R.

REFERENCES

1. **Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1995. Short protocols in molecular biology, 3rd ed., p. 4-20–4-22. John Wiley & Sons, Inc., New York, N.Y.
2. **Bassler, B. L.** 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. Curr. Opin. Microbiol. **2:**582–587.
3. **Bruckner, R.** 1992. A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. Gene **122:**187–192.
4. **Bruckner, R., O. Shosheyov, and R. H. Doi.** 1990. Multiple active forms of a novel serine protease from *Bacillus subtilis*. Mol. Gen. Genet. **221:**486–490.
5. **Chen, L., and J. D. Helmann.** 1994. The *Bacillus subtilis* σ^D-dependent operon encoding the flagellar proteins FliD, FliS, and FliT. J. Bacteriol. **176:**3093–3101.
6. **Ferrari, E., A. S. Jarnagin, and B. F. Schmidt.** 1993. Commercial production of extracellular enzymes, p. 917–937. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. Amercian Society for Microbiology, Washington, D.C.
7. **Fraser, G. M., and C. Hughes.** 1991. Swarming motility. Curr. Opin. Microbiol. **2:**630–635.
8. **Harshey, R. M.** 1994. Bees aren't the only ones: swarming in gram-negative bacteria. Mol. Microbiol. **13:**389–394.
9. **Harshey, R. M., and T. Matsuyama.** 1994. Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. Proc. Natl. Acad. Sci. USA **91:**8631–8635.
10. **Jones, L. J. F., R. Carballido-Lopez, and J. Errington.** 2001. Control of cell shape in bacteria: helical, actin-like filaments in *Bacillus subtilis*. Cell **104:** 913–922.
11. **Kawamura, F., and R. H. Doi.** 1984. Construction of *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. J. Bacteriol. **160:**442–444.
12. **Liu, H., and A. Bretcher.** 1989. Disruption of single tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. Cell **57:**233–242.
13. **Magnuson, R., J. Solomon, and A. D. Grossman.** 1994. Biochemical and genetic characterization of a competence pheromone from *B. sutbilis*. Cell **77:**207–216.
14. **Moran, C. P., Jr.** 1993. RNA polymerase and transcription factors, p. 653–667. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
15. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination, and outgrowth, p. 442–443. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus.* John Wiley & Sons, Inc., New York, N.Y.
16. **Samant, R. S.** 1998. Ph.D. thesis. Indian Institute of Technology, Mumbai, India.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 7.79–7.83. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Sloma, A., A. Ally, D. Ally, and J. Pero.** 1988. Gene encoding a minor extracellular protease in *Bacillus subtilis.* J. Bacteriol. **170:**5557–5563.
19. **Stahl, M. L., and E. Ferrari.** 1984. Replacement of the *Bacillus subtilis* subtilisin structural gene with an in vitro derived deletion mutation. J. Bacteriol. **158:**411–418.
20. **Steinmetz, M., and R. Richter.** 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through in vivo recombination. Gene **142:**79–83.
21. **Steinmetz, M., and R. Richter.** 1994. Easy cloning of mini-Tn*10* insertions from the *Bacillus subtilis* chromosome. J. Bacteriol. **176:**1761–1763.
22. **Stewart, B., J. L. Enos-Berlage, and L. L. McCharter.** 1997. The *lonS* gene regulates swarmer cell differentiation of *Vibrio parahaemolyticus.* J. Bacteriol. **179:**107–114.
23. **Strauch, M. A., and J. A. Hoch.** 1993. Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. Mol. Microbiol. **7:**337–342.